

The University of Chicago

**EFFECT OF HIGH MELANOPHORE
HORMONE FRACTIONS ON TYRO-
SINE AND DOPA OXIDATION**

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

1939

By

GERALD A. FOSTVEDT

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EFFECT OF HIGH MELANOPHORE HORMONE FRACTIONS ON TYROSINE AND DOPA OXIDATION^{1,2,3}

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THE INFORMATION concerning the rôle of the melanophore hormone of the pituitary body in higher animals is meager. Most of the studies with this hormone have been performed with lower animals in which the interest has centered around the action of the hormone on the melanophore and related cells of the dermis (1). The purpose of the present studies was an attempt to correlate melanophore hormone activity with melanin formation, and through these studies it was hoped to gain some insight into the possible rôle of this hormone in higher animals.

Teague (2) has given a concise review of this hormone, and further information may be obtained from other sources (3). Hence, references will be made only to the literature appertaining to the present investigation.

One of the best known processes of melanin formation is from tyrosine through the action of tyrosinase. Raper (4) has elaborated upon the exact mechanism of the oxidation of tyrosine by tyrosinase. Pigmentation in rabbits was studied by Onslow (5). In human skin Bloch (6) has identified the pigment forming enzyme as dopase, the substrate being dopa. Figge (7) found strong indirect evidence for tyrosinase existence in amphibians. He has recently obtained evidence to show that melanin formation is a reversible oxidation-reduction system, which may be as important as other redox systems in the body (8). In a preliminary report (9) experiments were cited by the writer in which certain pituitary fractions accelerated an oxidase system such as tyrosine-tyrosinase. This finding appeared to merit further investigation, since Collip and his coworkers (10) had reported the existence of a specific metabolic stimulant in fractions which contained a high concentration of melanophore hormone. However, Gaebler (11) had earlier recognized that metabolic stimulation was caused by anterior pituitary extracts. Teague (12) reinvestigated the possible metabolic stimulating effects of pituitary extracts. He found that such stimulation is not necessarily a specific response, since an increased oxygen consumption was noted when extracts of liver, muscle and kidney were injected into rats. Preparations of the pituitary gland rich in melanophore hormone but obtained from various sources and prepared by different methods varied considerably in their effect on the oxygen consumption of rats. Hypophysectomized or thyroidectomized rats gave the same variable response as did normal animals. In a more recent communication Teague (2) showed that the melanophore hormone and the specific metabolic stimulant are undoubtedly not identical, since the dose required for metabolic stimulation was 2000 times greater than that required for a marked melanophore response in hypophysectomized frogs, such a dosage being entirely out of the physiological range. A recent report (13) from Collips' laboratory is in essential agreement with Teague's conclusion.

¹ Aided by grants from the Rockefeller Foundation and the Committee on Research in Endocrinology of the National Research Council.

² Part I of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.

³ A preliminary report on some of the material given here has been published (*Proc. Soc. Exper. Biol. & Med.*, 40: 302, 1939).

From a survey of the literature one is justified in stating that there are at least four criticisms of the evidence thus far presented. First, there is disagreement as to the specificity of the metabolic stimulation produced by pituitary extracts, as evidenced by the work of Collip and coworkers (10) and of Teague (2). Secondly, there has been no definite indication that tyrosinase is present in mammals. Thirdly, there has been no report of an increase in melanin content of the frog's skin after the injection of melanophore hormone. Fourthly, the final outcome of any conclusive experimentation will be decided only when crystallized hormones become available.

EXPERIMENTAL STUDIES AND RESULTS

The experimental approach to the problem was through the use of a known oxidase system, namely the production of melanin through the action of tyrosinase on

TABLE I. METHOD OF PREPARATION OF FRACTIONS

Fraction I	Fraction II	Fraction III
1. 5 gm. pituitary powder in 0.25% acetic acid, boiled for 1 minute. 2. Filtered. 3. Filtrate evaporated to dryness in a current of air without heat. 4. Residue taken up with 50 cc. of methyl alcohol; insoluble portion centrifuged out. 5. 100 cc. of acetone added to the methyl alcohol solution. Precipitate is Fraction I. 6. Fraction I removed and dried on a watch glass in a desiccator. 7. When injected into a hypophysectomized frog, 1 cc. of a 10^{-5} fresh solution caused a marked darkening for 24 hours.	1. 5 gm. pituitary powder in 0.25% acetic acid, boiled for 1 minute. 2. Filtered. 3. Filtrate evaporated to dryness in a current of air without heat. 4. Residue taken up with 50 cc. of methyl alcohol; insoluble portion centrifuged out. 5. 5 cc. of N/10 HCl added to the methyl alcohol solution, then 150 cc. of acetone. Precipitate is Fraction II. 6. Fraction II removed and dried on a watch glass in a desiccator. 7. When injected into a hypophysectomized frog 1 cc. of a 10^{-6} freshly prepared dilution caused a marked darkening for 24 hours.	1. 5 gm. pituitary powder in 0.25% acetic acid, boiled for 1 minute. 2. Filtered. 3. Filtrate evaporated to dryness in a current of air without heat. 4. Residue triturated with 5 cc. of 1/10 N HCl; 50 cc. of methyl alcohol added; insoluble portion centrifuged out. 5. 150 cc. of acetone added to methyl alcohol and HCl solution. Precipitate is Fraction III. 6. Fraction III dried on a watch glass in a desiccator. 7. When injected into a hypophysectomized frog 1 cc. of 10^{-6} freshly prepared dilution caused a marked darkening for 24 hours.

tyrosine, and measuring the O_2 consumption in a Warburg apparatus. Changes in O_2 uptake were observed after the addition of various melanophore containing pituitary extracts, with adequate controls in all cases. The various procedures were as follows.

A. Several high melanophore hormone fractions were prepared by a modification of Stehle's method (14). By definition these fractions are referred to in this paper as high melanophore hormone fractions, because they give a marked physiological response in hypophysectomized frogs when injected in high dilutions, although it is not definitely known whether the melanophore hormone or some other pituitary principle is responsible for the effects on the oxidation systems studied. Table 1 shows the method of preparation of the fractions in an outline form. The fractions were prepared from acetone desiccated powder of a), anterior and b), posterior lobe beef pituitary glands;⁴ c), anterior lobes of sperm, finback and white whales; and d), from beef liver, lung, kidney and muscle. Different sources for the preparation of the extracts were used in order to insure fractions containing a different admixture of hormones.

⁴ Generously supplied by the Armour Laboratories and by the Wilson Laboratories, Chicago, Illinois.

B. The pituitary fractions, dissolved in distilled water, were assayed for their melanophore content by injecting them into the ventral lymph sac of hypophysectomized frogs. In preparations made from posterior pituitary powder it was also considered essential to measure the amount of pressor and oxytocic hormones which may act as contaminants. Figure 1 shows the pressor assay of these fractions.

C. The tyrosinase solution was made by triturating 2 gm. of meal worms in 10 cc. of buffer solution and centrifuging out as much extraneous material as possible. These tyrosinase solutions were crude and undoubtedly made up a part of the substrate; for this reason, more oxygen was consumed in these experiments than would theoretically be taken up. This point was checked by adequate controls. It should be especially noted and emphasized that the enzyme solutions varied in their activity, regardless of the fact that the same procedure was used in each instance. It was found that large meal worms usually gave

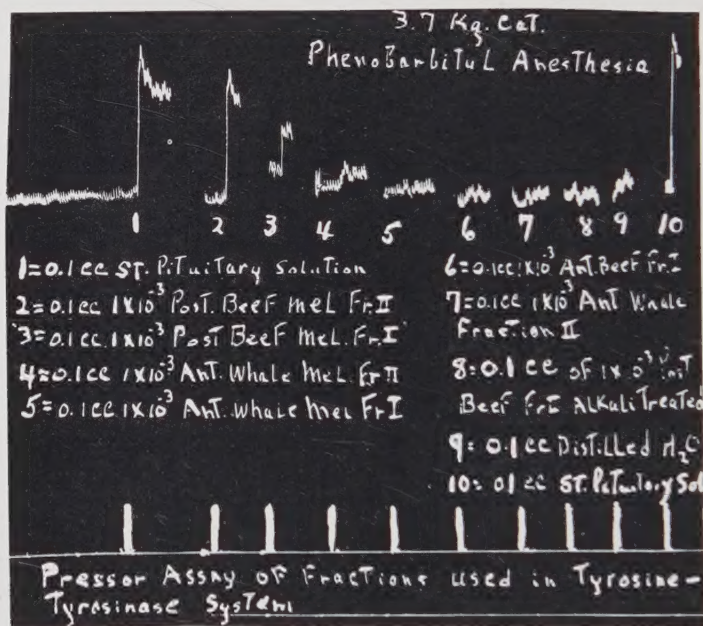


Fig. 1

a more potent enzyme solution while preparation of the enzyme from small meal worms resulted in a less active solution. Each experiment required its own enzyme system of control. The difference in enzymatic activity accounts for the variation in O_2 consumption from experiment to experiment. For example, in a very rapid oxidizing system due to high enzymatic activity only a small increase in the oxidation rate might be noted if a high melanophore hormone fraction was added to the enzyme-substrate system, whereas a greater increase in the oxidation rate was usually noted if the activity of the enzyme was weaker. This fact must constantly be borne in mind when interpreting any apparent discrepancies between experiments. The most consistent results were obtained when the enzyme activity was such that 25 to 50 cu. mm. of oxygen was consumed per hour. A slow reaction was obtained by conducting the experiments in a slightly acid media. There is often an initial latent period in the enzyme activity, a fact well known to workers in the field.

D. The substrate used was recrystallized tyrosine. It was dissolved in a sodium dihydrogen phosphate-sodium hydroxide buffer at a pH of 6.85 in most experiments, and at a pH of 7.33 in others. Heating in a boiling bath was necessary to bring all the tyrosine into solution. The tyrosine solution, in addition to the enzyme and melanophore containing

solutions, was freshly prepared before each experiment. Most of the tests were conducted at a pH 6.85 since the activity of the enzyme is slower in this range. In these experiments 1.5 cc. of 0.0033 molar tyrosine solution, 0.3 cc. of tyrosinase solution, and 0.3 cc. of a 1/1000 or 1×10^{-3} dilution of a high melanophore hormone fraction were used, with modifications of the total substrate only in the case of the tryptic digestion experiments.

E. The Warburg apparatus (15) with the two side arm vessels was used. The substrate was placed in the bottom of the vessel, while the solution of enzyme and the high melanophore hormone fraction were each placed in their respective side arms. After reaching a constant temperature of 38° C. the contents of the side arms were tipped into the substrate solution as quickly as possible and readings of the O₂ uptake were taken every 15 minutes or oftener. All experiments were run in duplicate with controls in each case.

F. In an experiment to determine the extent to which a high melanophore hormone

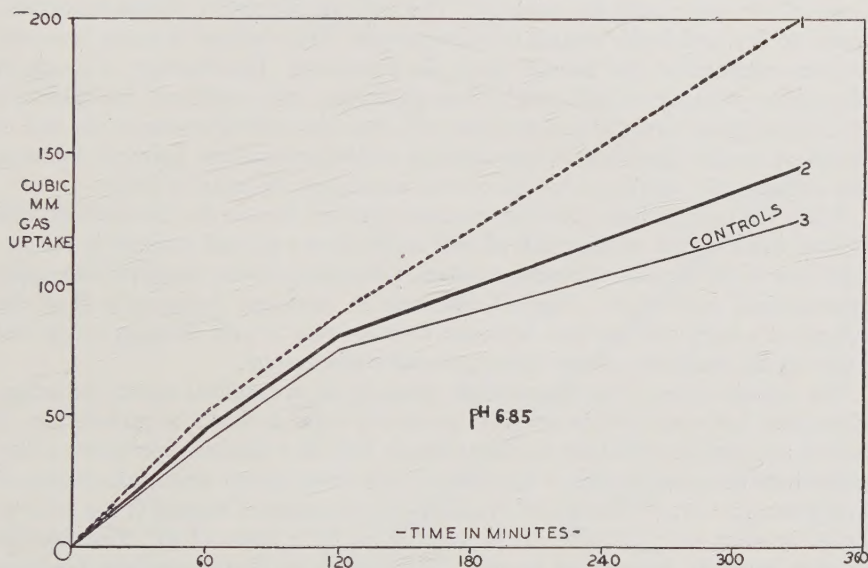


Fig. 2. EFFECT OF POSTERIOR LOBE BEEF MELANOPHORE ON TYROSINE-TYROSINASE SYSTEM. Curve 1, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. Fraction II posterior lobe beef melanophore (10^{-3} alkali-treated). Curve 2, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. Fraction II posterior lobe beef melanophore (10^{-3} untreated). Curve 3, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. buffer.

fraction could be diluted and still have an effect on the tyrosine-tyrosinase system, it was found that any concentration less than 140 γ in the total volume resulted in a decreased effect on this oxidase system. This experiment was tried with posterior beef pituitary high melanophore hormone Fraction I, in which the pressor and oxytocic hormones were not destroyed. Hence it was considered desirable to utilize at least this concentration of the fractions in these experiments.

High melanophore hormone Fraction II, prepared from the posterior lobe of beef pituitary, was found by assay to contain considerable pressor and oxytocic activity (fig. 1). Therefore, this activity was destroyed by heating with 2 N sodium hydroxide for 1 hour in a boiling water bath, then neutralizing with 4 N hydrochloric acid, and finally diluting with buffer solution until the high melanophore hormone fraction was present as a 1×10^{-3} dilution. (fig. 2, curve 1). Determinations on this preparation were compared with those on buffered melanophore containing extract of the same strength which had not previously been treated with alkali and acid (fig. 2, curve 2). Both of these determinations were compared with the O₂ uptake of samples containing only the substrate tyrosine, the enzyme tyrosinase and the buffer (fig. 2, curve 3).

From figure 2 it is seen that with a high melanophore hormone fraction whose oxytocic and pressor content was destroyed by treatment with alkali there was a marked increase (60%) in O_2 uptake as compared to the control value for the substrate-enzyme system (curve 3). The melanophore hormone fraction accelerated, possibly catalytically, tyrosine-tyrosinase oxidation. The untreated preparation (curve 2) showed only a 16% increase in O_2 consumption by the end of $5\frac{1}{2}$ hours, indicating the marked inhibitory influence of the pressor and oxytocic substances.

In order to exclude the possibility of autolysis as a factor, freshly removed pituitary glands were obtained at the Armour Laboratories and immediately frozen in dry ice. They were brought to the laboratory and allowed to thaw, and the anterior and posterior lobes were carefully separated. The anterior lobes were placed in acetone, allowed to dry, and finally ground to a fine powder. This acetone desiccated powder was then extracted in the manner given for Fraction I. This fraction was rich in melanophore principle as indicated by frog injections, and accelerated the oxidation rate of the tyrosine-tyrosinase system by 30% over the control system at the end of 6 hours. A similar experiment using anterior whale melanophore hormone Fraction I also showed 30% increase over the control system by the end of 6 hours.

While the melanophore hormone fractions obtained from either the anterior lobe of sperm whale or the anterior lobe of beef cattle show a marked increase in the oxidation rate of the tyrosine-tyrosinase system, injections of these fractions into hypophysectomized frogs elicit a marked difference in response. Apparently then the presence of a high melanophore hormone fraction over a wide dilution range will accentuate the oxidation rate of the enzyme-substrate system.

The anterior lobe of the sperm whale pituitary is an excellent source for a high melanophore hormone fraction and is of particular value in that it is entirely free of oxytocic and pressor principles as contaminants (16). In a further experiment a high melanophore hormone fraction of the anterior lobe of the sperm whale was destroyed by subjecting a 1×10^{-3} dilution of Fraction I to the action of trypsin (1 mg. of 1-75 powder in 10 cc. of 7.33 buffer). It was incubated for 2 hours at 38° . The solution was then boiled to destroy the trypsin. The activity of the melanophore hormone fraction was completely destroyed, as determined by injections into hypophysectomized frogs. The activity of the tyrosine-tyrosinase system containing the inactivated melanophore hormone fraction is shown by curve 2 of figure 3. Curve 1 of figure 3 represents the activity of such a system containing an active melanophore hormone fraction made up in a portion of inactivated trypsin solution. In addition, this was compared with a system containing tyrosine-tyrosinase plus the inactivated trypsin solution described above, the latter being added instead of buffer alone, since the substrate would then be the same in all the samples. The latter system acted as a control and is represented by curve 3 of figure 3.

This experiment indicates clearly that a high melanophore hormone fraction does not act as additional substrate for this system, since when the hormone is destroyed, the presence of the inactivated melanophore hormone fraction does not accelerate the O_2 uptake over the control. The active high melanophore hormone fraction causes an acceleration of the system, in this case 34%. This experiment was conducted at pH 7.33 and shows further that the accelerating activity of the high melanophore hormone fraction occurred in an alkaline as well as in the acid range. Another experiment with high melanophore hormone Fraction I from posterior beef pituitary gave essentially similar results at pH of 7.33. A subsequent experiment using only a high melanophore hormone fraction as a substrate for tyrosinase proved to be nega-

tive, again indicating that the melanophore fraction was not acting as added substrate in the experiments.

A high melanophore hormone, Fraction II, from the anterior lobe of sperm whale was tested for its activity on the tyrosine-tyrosinase system. It had greater activity than any of the preparations made from the anterior lobe of sperm whale pituitary. The same concentration of tyrosine, tyrosinase and hormone were used as in previous experiments, at a pH of 6.85. A high melanophore hormone fraction accelerated the oxidation rate 120% by the end of a 4-hour experiment. This experiment proved to be an exception since subsequent experiments have shown a wide range of accelera-

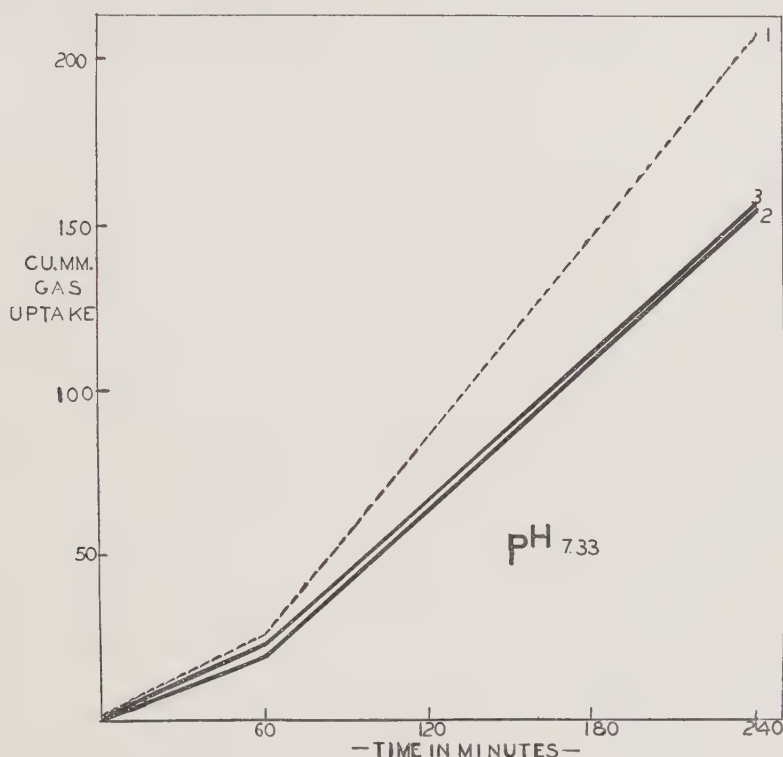


Fig. 3. EFFECT OF INACTIVATION OF MELANOPHORE. Curve 1, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. of 10^{-8} anterior lobe whale Fraction I in inactive trypsin. Curve 2, same as curve 1 except melanophore was destroyed by trypsin. Curve 3, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. of inactive trypsin in buffer.

tion, namely, 23, 28, 35, 40, 60 and 80% increases, respectively. These variations are chiefly a result of the enzyme variability described previously.

The results obtained with high melanophore hormone Fraction II of the anterior lobe of the pituitary of sperm whale suggested further experimentation to determine what effect the presence of pressor and oxytocic principles would have on the oxidation process. To do this the tyrosine-tyrosinase system was tested with Fraction II obtained from the anterior lobe of sperm whale and made up in a buffer solution. The control system had Fraction II of the anterior sperm whale dissolved in standard posterior pituitary solution (containing the pressor and oxytocic hormones).

Adding standard beef posterior pituitary solution had a definite inhibitory effect

(only 24% increase, curve 2) as compared with the system which contained a high melanophore hormone fraction alone in buffer (56% increase, curve 1). Both showed an increase in oxidation rate over the enzyme-substrate-buffer control (curve 3) because of the presence of a high melanophore hormone fraction.

Further information as to the inhibitory nature of the pressor and oxytocic hormone was obtained when it was found that 0.3 cc. of an acetic acid extract of the neural lobe of the sperm whale, which contains pressor and oxytocic hormones, but no melanophore hormone, decreased the oxidation rate of this oxidase system 12% as compared to the controls by the end of a 3-hour period.

In a tyrosine-tyrosinase-high melanophore hormone fraction system the hormone is partially or wholly destroyed during the oxidation of the substrate. This was de-

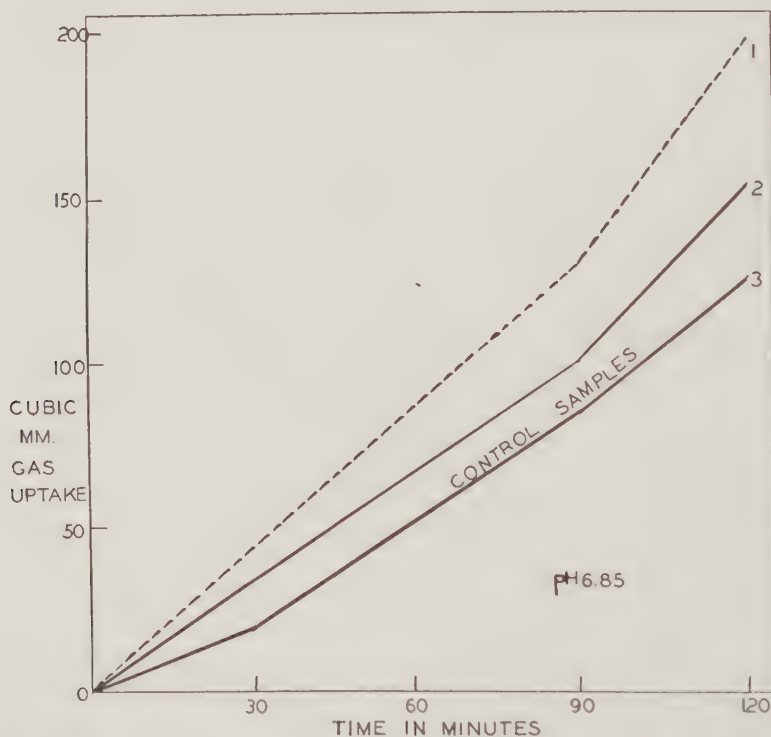


Fig. 4. EFFECT OF ADDITION OF POSTERIOR BEEF STANDARD PITUITARY SOLUTION. Curve 1, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. 10^{-8} Fraction II anterior sperm whale melanophore (in buffer). Curve 2, same as curve 1 except Fraction II is dissolved in standard pituitary solution. Curve 3, 1.5 cc. of 0.0033 molar tyrosine, 0.3 cc. tyrosinase, 0.3 cc. buffer.

termined by injecting the resulting melanin product into hypophysectomized frogs. The addition of 8-hydroxy quinoline to the system prevents the oxidation even when a high melanophore fraction is present.

As a further control in these experiments, and to determine the specificity of the melanophore fraction, Fractions I, II and III were prepared from beef muscle, liver, kidney and lung tissue in place of pituitary powders. Inclusion of these fractions in the tyrosine tyrosinase system in the same manner as the melanophore fractions resulted in no appreciable increase in O_2 uptake in the oxidase system. In fact, they checked closely with the controls.

Dihydroxyphenylalanine, or dopa, is the first product formed in the oxidation of

tyrosine. Dopa itself is slowly autoxidizable in the alkaline range at a temperature of 38° C. When one includes sperm whale high melanophore hormone Fraction II in such a system with the same concentrations of substrate and hormone as in the tyrosine-tyrosinase experiments the effect is an inhibition of the oxidation rate of dopa as compared to the controls. This is exactly contrary to expectations when one considers the effects of this fraction on the tyrosine-tyrosinase system. The decrease in the oxidation rate was highest between the third and fourth hours, a decrease of 55% being manifested at this point. At the end of the experiment the resulting melanin solution was injected into the hypophysectomized frog and the hormone was found to be partially or wholly destroyed.

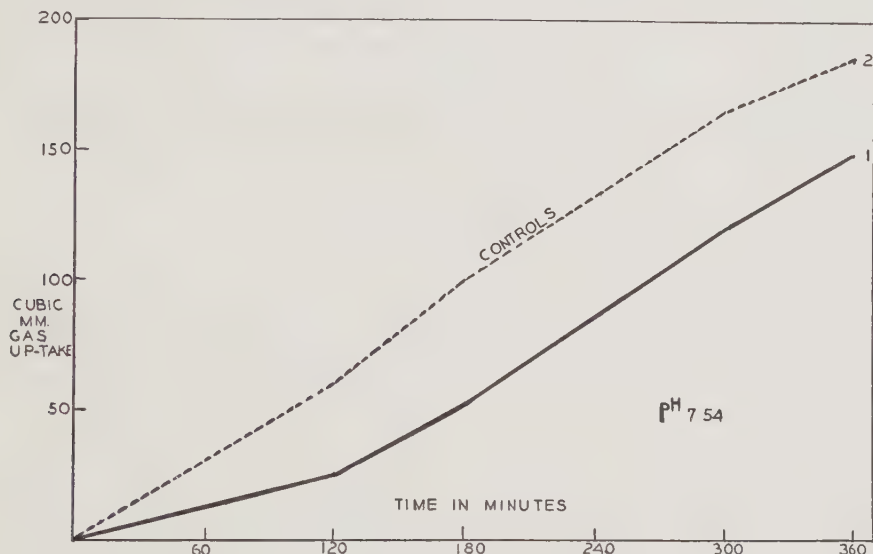


Fig. 5. EFFECT OF FRACTION III FROM ANTERIOR LOBE OF FINBACK WHALE ON DOPA OXIDATION. Curve 1, 1.8 cc. of 0.0033 molar dopa, 0.3 cc. of 10^{-3} finback Fraction III. Curve 2, 1.8 cc. of 0.0033 molar dopa, 0.3 cc. of buffer.

A high melanophore hormone Fraction III prepared from the anterior lobe of finback and white whale pituitary gland had an effect quite opposite to that of Fractions I and II on the oxidase system. Fraction III differs essentially in that the dried filtrate from the acetic acid extraction of the pituitary gland is triturated with 5 cc. of 1/10 normal hydrochloric acid. This fraction caused a 22% decrease in tyrosine-tyrosinase oxidation as compared to the control samples. Thus appeared the first evidence of the presence of a substance extracted from pituitary gland, other than the pressor and oxytocic substances, which would cause a decrease in the rate of oxidation of an oxidase system. Furthermore, Fraction III exhibited an even more pronounced inhibition of dopa oxidation. The maximum inhibition was at the end of the second hour, when a decrease of 140% was observed (fig. 5).

The inhibition of a slowly oxidizing substance such as dopa by high melanophore hormone Fractions II and III, however, was not specific for pituitary gland extracts. Fractions II and III prepared from lung, liver, kidney and muscle also inhibited dopa autoxidation, but to a much less degree, possibly acting only as contaminants. The maximal inhibition of dopa oxidation by tissue fractions was as follows.

(a) Muscle Fractions II and III showed 8% inhibition by the 6th hour.

- (b) Liver Fractions II and III showed 20% inhibition by the 6th hour.
- (c) Lung Fraction III showed 13% inhibition by the fourth hour.
- (d) Fraction I of these organs had little or no effect.

The experiments up to this point appear to suggest that there exists a substance in pituitary fractions high in melanophore hormone activity which accelerates the oxidation of tyrosine by tyrosinase. However, if these pituitary fractions are prepared as Fraction III, an inhibition of tyrosine-tyrosinase oxidation occurs, as well as a marked inhibition of slowly autoxidizable dopa solutions. Extracts made from other organs in a manner similar to the pituitary fractions do not have an effect on tyrosine-tyrosinase oxidation. However, extracts of other organs did inhibit dopa oxidation but to a minor degree when compared with Fraction III prepared from whale anterior pituitary glands. That two such diametrically opposite effects should be obtained with dopa and tyrosine oxidation is difficult to explain on the basis of the kinetics of the reaction elaborated by Raper. The substances responsible for these diverse activities were obtained by changing the extraction procedures, as indicated by Fraction II and III in table 1. Experimental procedures are now in progress in an attempt to further differentiate the activities of the fractions.

DISCUSSION

The results reported here show that high melanophore hormone Fractions I and II increased the rate of oxidation of a tyrosine-tyrosinase system. This oxidase system was inhibited by the presence of pressor and oxytocic hormones, as well as by Fraction III from anterior lobes. Figge's (7) indirect evidence for the existence of tyrosinase in amphibian larvae would be in harmony with the accelerator effect of a high melanophore hormone fraction on the oxidation of a tyrosine-tyrosinase system in *in vitro* experiments.

Several possibilities appear evident. A hormone appears to be present which accelerates enzymatic oxidation such as that of tyrosinase. Another substance is present in these fractions which inhibits the self-oxidation of dihydroxyphenylalanine. However, both the depressant from the pituitary and the other organ extracts decrease the self-oxidation of dopa, the organ extracts to a much less degree than Fraction III prepared from anterior pituitary lobes. Also it is conceivable that the inhibitory factor may have been formed during the extraction.

The experiments showing the acceleration of an oxidase system by the addition of dilutions of high melanophore hormone fractions agrees with the results of Collip and his coworkers who observed metabolic stimulation as a result of injection of various pituitary extracts into operated animals. However, there are other considerations to be made which do not simplify any conclusions which might be drawn.

Teague (12) obtained a significant increase in O_2 consumption in 18 of 45 experiments when operated rats were injected with extracts rich in melanophore hormone. However, no significant change was found in 27 experiments, and in a few experiments there was actually a decrease in metabolism. In general he had to inject very large doses to obtain a significant metabolic increase, doses which were entirely out of the physiological range for the melanophore hormone. The marked decrease in the autoxidation of dopa and of tyrosine tyrosinase oxidation with Fraction III may account for Teague's results, in which he actually obtained a decrease in metabolism of some animals and no significant change in 27 of 45 experiments, if one is permitted to translate results of *in vitro* experimentation to animal experimentation.

SUMMARY

High melanophore hormone fractions (I, II, III) have been obtained which caused a marked pigment dispersion for 24 hours after the injection of 1 cc. of a 1×10^{-6}

dilution into the hypophysectomized frog. By definition these fractions are referred to as high melanophore hormone fractions because they give a marked physiological response in hypophysectomized frogs when injected in high dilutions.

Fractions I and II produced a marked acceleration of the oxidase system tyrosine-tyrosinase.

Fraction III which was prepared from the anterior lobe of the finback and the white whale pituitary caused a marked decrease (140%) in dopa autoxidation, as well as tyrosine-tyrosinase oxidation (22%). The possibility of the formation of this inhibitor during chemical manipulation must be considered.

The pressor hormone and, perhaps, the oxytocic hormone appear to inhibit the oxidase system tyrosine-tyrosinase. However, a fraction has been obtained from the anterior pituitary lobe which also caused an inhibition of tyrosine-tyrosinase oxidation. It may be this factor, present in both lobes, which inhibits the oxidative process, as well as the pressor and oxytocic hormones.

Liver, kidney, lung and muscle extracts had no effect on the tyrosine-tyrosinase system. These same extracts had much less inhibitory effect on dopa oxidation than did the pituitary fractions.

The author wishes to express his appreciation for advice and criticism to Dr. E. M. K. Geiling of the Department of Pharmacology under whose direction this work was completed, and to Dr. Barron of the Lasker Foundation of the University of Chicago, who extended the facilities of his laboratory for a part of this research.

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